The zinc transporter ZIP12 regulates the pulmonary vascular response to chronic hypoxia

Extended Data

Extended Data Figure 1. Generation of congenic and sub-congenic strains. Congenic rat lines were produced by introgression of the F344 chromosome 17 QTL segment onto the WKY genetic background by repeated backcrossing. Congenic rat strain R47A (WKY.F344-D17Got91/D17Rat51) contains 15Mbp from the F344 donor region that maps to the distal end of the QTL on a WKY background. Three sub-congenic strains, SubA (WKY.F344-D17Got91/D17Rat47), SubB (WKY.F344-D17Rat47/D17Rat51) and SubC (WKY.F344-D17Rat131/D17Rat51), were produced containing separate fragments of the R47A donor region by backcrossing of (R47A x WKY) F1 with WKY parental rats. Three recombination events within the R47A congenic interval break the congenic interval into three smaller and overlapping sub-congenic intervals (Figure 1, main text).

Extended Data Figure 2. Dissection of QTL and Cardiovascular phenotype of rat strains. a. An illustrative genetic map showing the relationship of the congenic strains (R42, R47A), subcongenic strains (SubA, SubB, SubC) and Slc39a12 to the original QTL (defined by a LOD score >3; Zhao et al Circulation 2001,103, 442-447) on a physical map of chromosome 17 (using Rat Genome Assembly v5.0). b. The hypoxia-resistant F344 phenotype tracks with the congenic R47A line. Rats were kept in 10%O2 for 2 weeks and right ventricular hypertrophy (RV/LV+Sep) was significantly attenuated in the congenic R47A strain (0.32±0.03, n=13, **P<0.01) compared to WKY rats (0.37±0.03, n=15), whereas congenic R42 rats (0.36±0.03, n = 17) were similar (NS) to WKY rats. In normoxia, WKY, F344, R47A, SubA, SubB and SubC rats show no significant differences in c. mean pulmonary artery pressure (mPAP), d. right ventricular hypertrophy (RV/LV+Septum ratio) and e. vascular muscularisation (n=8 each group); f. Systemic blood pressure (SBP) is similar in all strains in both normoxia and hypoxic conditions. g. F344, R47A and SubB rats exhibit attenuated pulmonary vascular remodelling after 2 weeks exposure to a 10% O2 atmosphere compared to WKY, SubA and SubC rats (n=6 each group). Values are expressed as the mean ± standard error of the mean (SEM). *P<0.05, **P<0.01, ***P<0.001 compared to WKY (% of fully muscularised and partially muscularised vessels); ##P<0.01, ###P<0.001 compared to

WKY (% of non-muscularised vessels) after One-Way ANOVA analysis followed by Bonferroni correction for multiple testing.

Extended Data Figure 3. Hypoxia-induced pulmonary vascular remodeling in parental strains. a. Upper panel sequence shows the WKY protein sequence (688aa) and lower panel shows the truncated F344 protein sequence (553aa). Stars (*) mark the mutated amino acids compared to WKY protein. Dotted line indicates the C-terminal truncated region in F344. The grey square highlights the metalloprotease motif. b. Prominent ZIP12 immunostaining is seen in remodelled pulmonary arterioles in the chronically hypoxic WKY rat alongside vessels with a double elastic lamina (stained with Van Gieson, EVG) but not F344 lungs exposed to hypoxia. (Red arrow: vessel with double elastic lamina; blue arrow: vessel with single elastic lamina)

Extended Data Figure 4. ZIP12 upregulation in response to hypoxia exposure and measurements of intracellular labile zinc concentration and proliferation of human pulmonary artery smooth muscle cells (HPASMCs) in normoxia conditions. a. Upregulation of ZIP12 in HPASMCs exposed to hypoxia, in contrast to other zinc transporters (n=6). b. Representative western blots demonstrating increased HIF-2α expression in HPASMCs after 24h hypoxia exposure. c. Confocal laser scanning images of HPASMC transfected with eCALWY-4 probe. Intracellular free zinc was not affected by transfection with ZIP12 siRNA in normoxia. d. Representative traces showing the changes in fluorescence ratio usng the eCALWY-4 probe. e. Quantification of intracellular zinc levels (n=10). f. ZIP12 siRNA did not affect proliferation of HPASMCs in normoxic conditions (n=5).

Extended Data Figure 5. Design of specific Slc39a12 ZFN and confirmation of mutant line. a. CompoZr™ Custom Zinc Finger Nucleases (Sigma-Aldrich) for the rat Slc39a12 gene were designed to target exon 8 (a; Sigma-Aldrich). b-d. Cel-I surveyor assay and gene sequencing confirmed NHEJ-induced mutations in at least one pup (mutant 77). e. The 4bp (AGTT) deletion followed by 2bp insertion (TA) into mutant 77 caused a frame-shift in coding, introducing a stop codon leading to a truncated protein. Red star refers to stop codon. c. We subsequently genotyped

next generation litters using SwaI (cutting point: 5'-ATTTAAAT-3'), showing 100% digestion for homozygous pups (-/-), 50% for heterozygous (+/-) and no DNA digestion for wild type rats (+/+).

Extended Data Figure 6. ZIP12 knockout attenuated hypoxia-induced pulmonary vascular remodelling. a. Representative lung sections from wild-type (WT) and ZIP12 -/- rats 2 weeks after hypoxia exposure. Elastic van Gieson (EVG) staining showing double elastic lamina (red arrow) in WT but single elastic laminae (blue arrow) in ZIP12-/- rats. b. Genetic disruption of ZIP12 in WKY rat attenuated pulmonary vascular remodelling after 2 weeks exposure to a 10% O2 atmosphere compared to wild-type (WT) rats (n=5 each group). *P<0.01 compared to WT (% of fully muscularised vessels); ##P<0.01, ###P<0.001 compared to WT (% of non-muscularised vessels) after One-Way ANOVA analysis followed by Bonferroni's multiple comparison test. c. Ki67 staining showing reduced proliferation in hypoxic ZIP12-/- rat lungs compared to the WT strain. *P<0.01 compared to WT. d. Representative sections from hypoxic WT and ZIP12-/- rats lungs showing differences in staining with the proliferation marker, Ki67. e-g. Genetic disruption of ZIP12 in WKY rat did not influence e. systemic blood pressure (SBP) or f. cardiac output (CO) but attenuated hypoxia-induced increases in g. pulmonary vascular resistance (n=7 each group). Values are expressed as the mean ± standard error of the mean (SEM). *P<0.05, **P<0.01 compared to normoxic rats, #p<0.05 compared to wild-type (WT) hypoxic rats after One-Way ANOVA analysis followed by Bonferroni correction for multiple testing. h. ZIP12 targeted siRNA inhibition attenuates stress fibre formation in human pulmonary vascular smooth muscle cells (HPASMCs) in hypoxia (n=5 each group). **p<0.01 compared to normoxia control group, #p<0.05 compared to hypoxia control group. i. Representative pictures of actin stress fibre in HPASMCs. j. ex vivo angiogenesis studies demonstrated that vascular outgrowth from ZIP12-/- pulmonary vessels in response to hypoxia was attenuated compared to vessels from wild-type (WT) rats (n=12 each group, 2 rings/rat, 6 ZIP12 -/- and 6 WT rats). *P<0.05 compared to normoxia WT group; #P<0.05, ## P<0.01 and ### P<0.001 compared to hypoxia ZIP12-/- group. k. Representative pictures of pulmonary arteriole ring outgrowth at day 6.

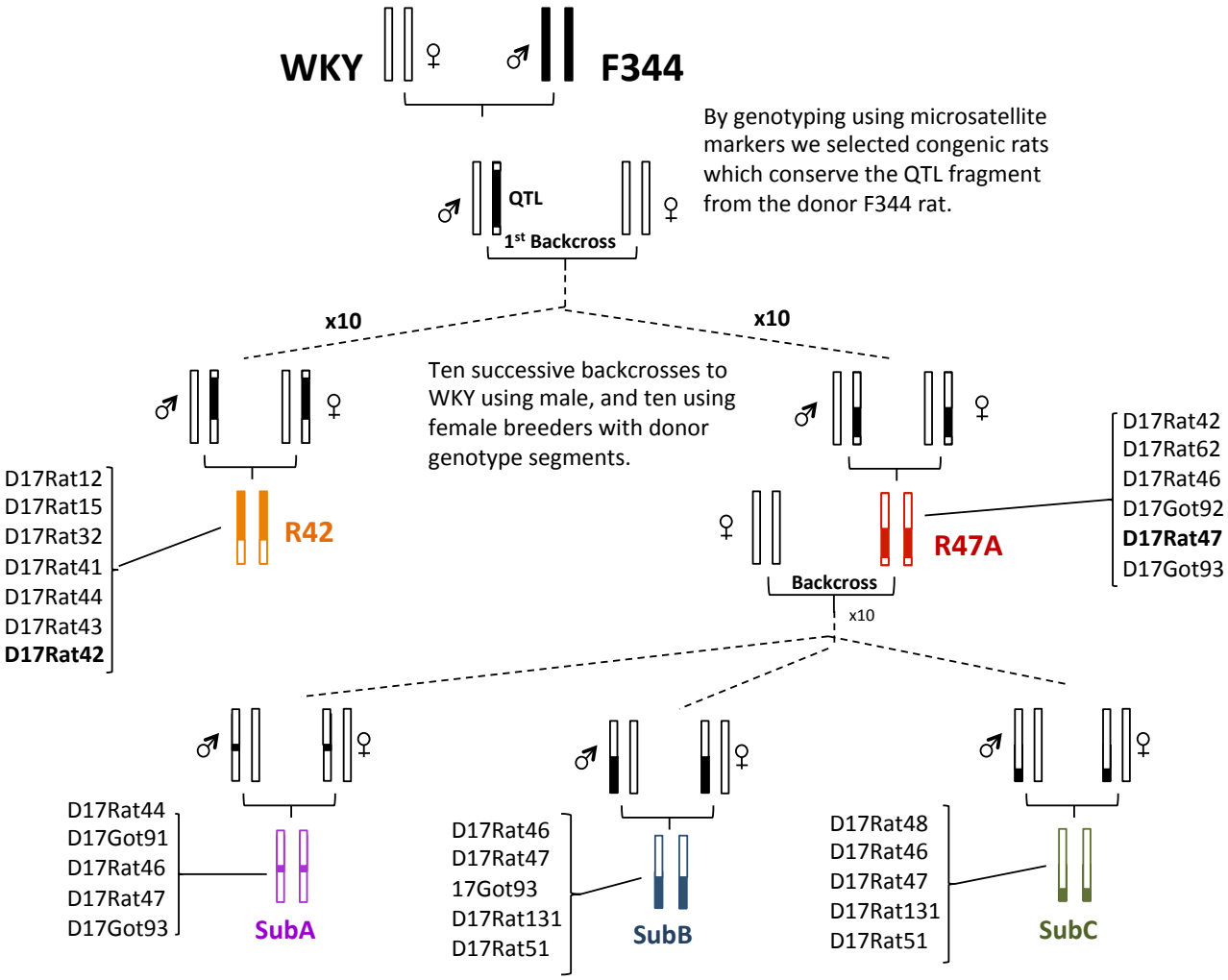
Extended Data Figure 7. Carbonic anhydrase (CAIX) expression. a. Representative sections demonstrating increased CAIX expression in remodelled pulmonary arterioles in the lungs of rats exposed to alveolar hypoxia (2 weeks), monocrotaline (MCT, 3 weeks) or a chronic iron deficient diet (4 weeks). b-c. No CAIX staining was detected in pulmonary arteries of low altitude (normoxia control, CO calf) calves and sea level humans, but prominent CAIX immunostaining was observed in the remodelled pulmonary arteries of calves with severe pulmonary hypertension (Hx calf), in cattle with naturally occurring pulmonary hypertension ("Brisket disease", BD) as well as patients with idiopathic pulmonary arterial hypertension (IPAH).

Extended Data Figure 8. Genetic disruption of ZIP12 in WKY rat attenuated monocrotaline-induced pulmonary hypertension. a. Mean pulmonary artery pressure (mPAP), b. right ventricular hypertrophy (RV/LV+Septum) and c. pulmonary arteriole muscularisation. (n=5 each group). Values are expressed as the mean ± standard error of the mean (SEM). *P<0.05, **P<0.01 compared to wild-type (WT) monocrotaline group after unpaired Student t-test. d. Representative lung sections from wild-type (WT) and ZIP12 -/- rats 3 weeks after monocrotaline injection. Elastic van Gieson (EVG) staining showing double elastic lamina (red arrow) in WT but single elastic laminae (blue arrow) in ZIP12-/- rats.

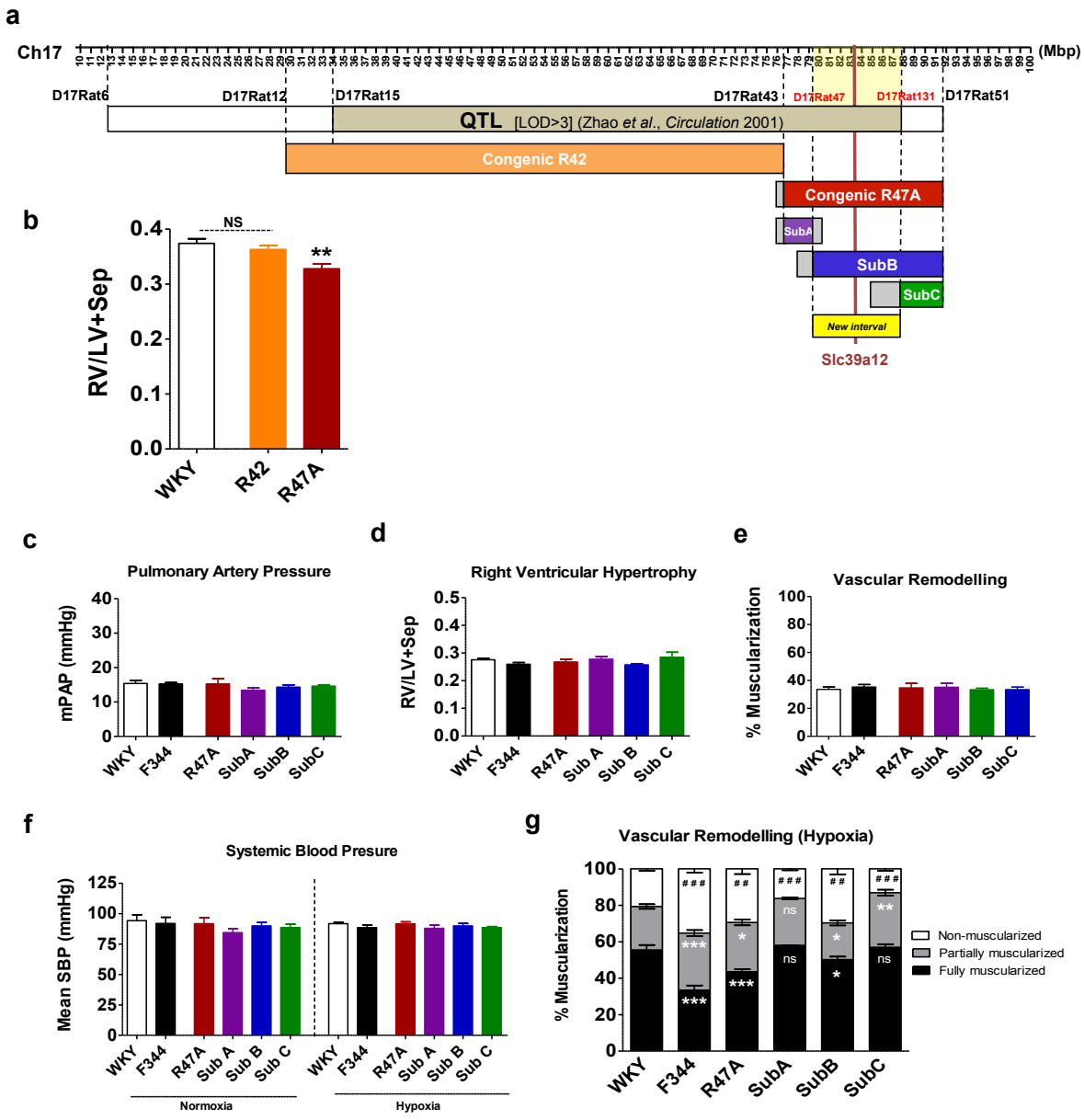
Extended Data Table 1. Frameshift and Non-Synonymous coding mutations in the refined congenic interval of F344 and the other hypoxia susceptible strains, WKY, spontaneously hypertensive (SHR) and fawn-hooded (FHH) rat strains.

Extended Data Table 2. Polymorphism markers for congenic strain genotyping.

Extended Data Figure 1.



Extended Data Figure 2.



Extended Data Figure 3.

a

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1       MCFWTELSVWVVLVSHSFSLASSTETSKALTQDNSRVGSHSLLEVLRVLSAGDDRSLNHP   60
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61      QSLIKILLERTGCPQRTDGTQEDCKLCLEPDALSLTAGGDLEDELREEVVQRVSLLLLYY   120
        QSLIKILLERTGCPQRTDGTQEDCKLCLEPDALSLTAGGDLEDELREEVVQRVSLLLLYY

121     IIHQEEICSSKLNMSNREYEFYLHSLLSLRQDEDSYFLSEKETDDILASTRKYFGTSTSP   180
        IIHQEEICSSKLNMSNREYEFYLHSLLSLRQDEDSYFLSEKETDDILASTRKYFGTSTSP

181     CMETKILQRESGIQSSNGADEKMLPQLAATIIALSLQGVCLGRKDSPSPDDFTEYIFSFL   240
        CMETKILQRESGIQSSNGADEKMLPQLAATIIALSLQGVCLGRKDSPSPDDFTEYIFSFL

241     NRTNALHLSELEELLNMLSTRRACTKINTLHEHQRKQNTAVHGLRDPKSAAAMDKVSGDH   300
        NRTNALHLSELEELLNMLSTRRACTKINTLHEHQRKQNTAVHGLRDPKSAAAMDKVSGDH

301     SVSWDQACFSAQQLVEIFLQNHSSLSISKEDFKQLSPGIIQQLLSCSCQVPRDQKAKPPP   360
        SVSWDQACFSAQQLVEIFLQNHSSLSISKEDFKQLSPGIIQQLLSCSCQVPRDQKAKPPP

361     TTLEKYGYSTVAVTLLTLGSMLGTALVLFHSCEENYSLILQLFVGLAVGTLSGDALLHLI   420
        TTLEKYGYSTVAVTLLTLGSMLGTALVLFHSCEENYSLILQLFVGLAVGTLSGDALLHLI

421     PQVLGLHKQEAELGHFHESQSPIWKLLGLLGGIHGFFLIEKCFILLVSPNTKGLPLVNGH   480
        PQVLGLHKQEAELGHFHESQSPIWKLLGLLGGIHGFFLIEKCFILLVSPNTKGLPLVNGH

481     AGHTHHLGLSPELNDQSGGGKSISTIQLKGPEDSQTAELPKGNVPASNRNRKTISLLAVM   540
        AGHTHHLGLSPELNDQSGGGKSISTIQLKGPEDSQTAELPKGNVPASNRNRKTISLLAVM

541     VLVGDGLHNFADGLVIGTAFSSSLESGVTTTIAILCHEIPHEMGDFAVLLSSGLSIRTAI   600
        VLVEMACTILPMA.............................................|
           **********
601     LMNFLSALTAFIGLYIGLSVSADPRVQDWILTVTAGMFLYLSLVGMLPEMTHVQTQRPWM   660
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661     TFLLQNVGLVLGWFSLLLLAVYEQNIKI.   688
        ............................
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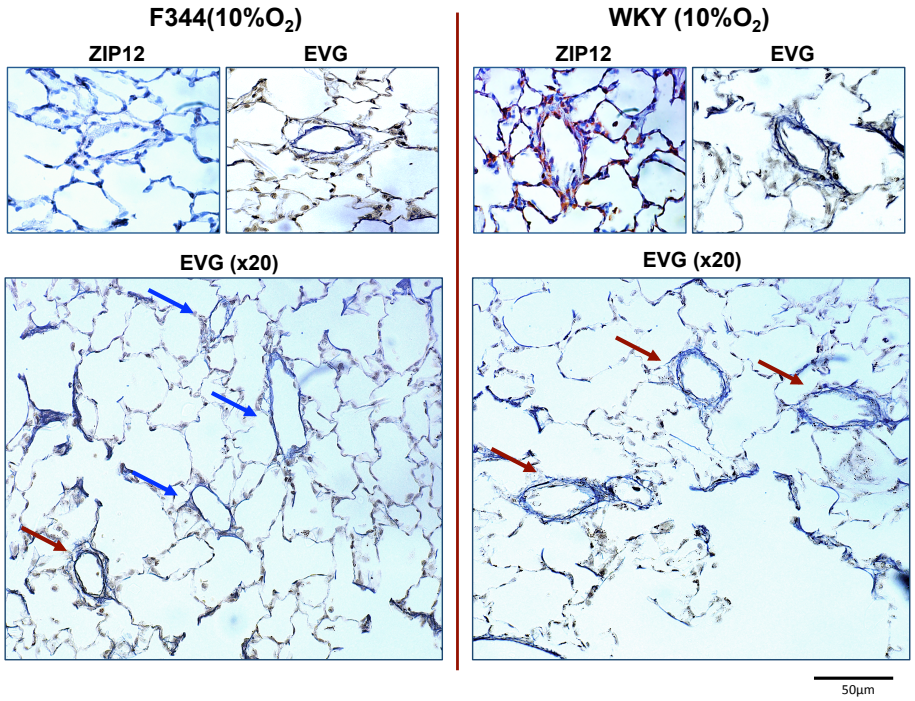
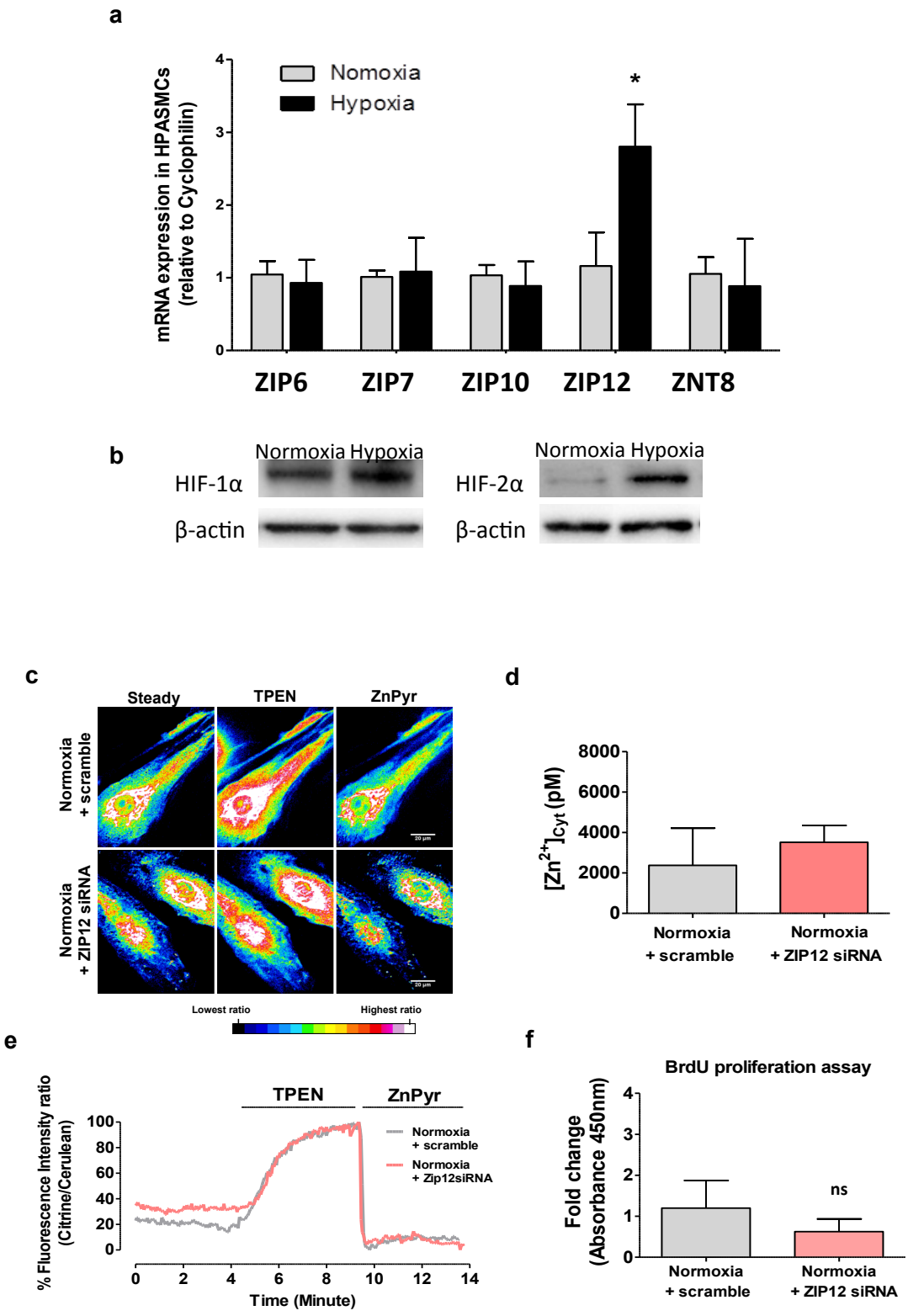
b



Extended Data Figure 4.



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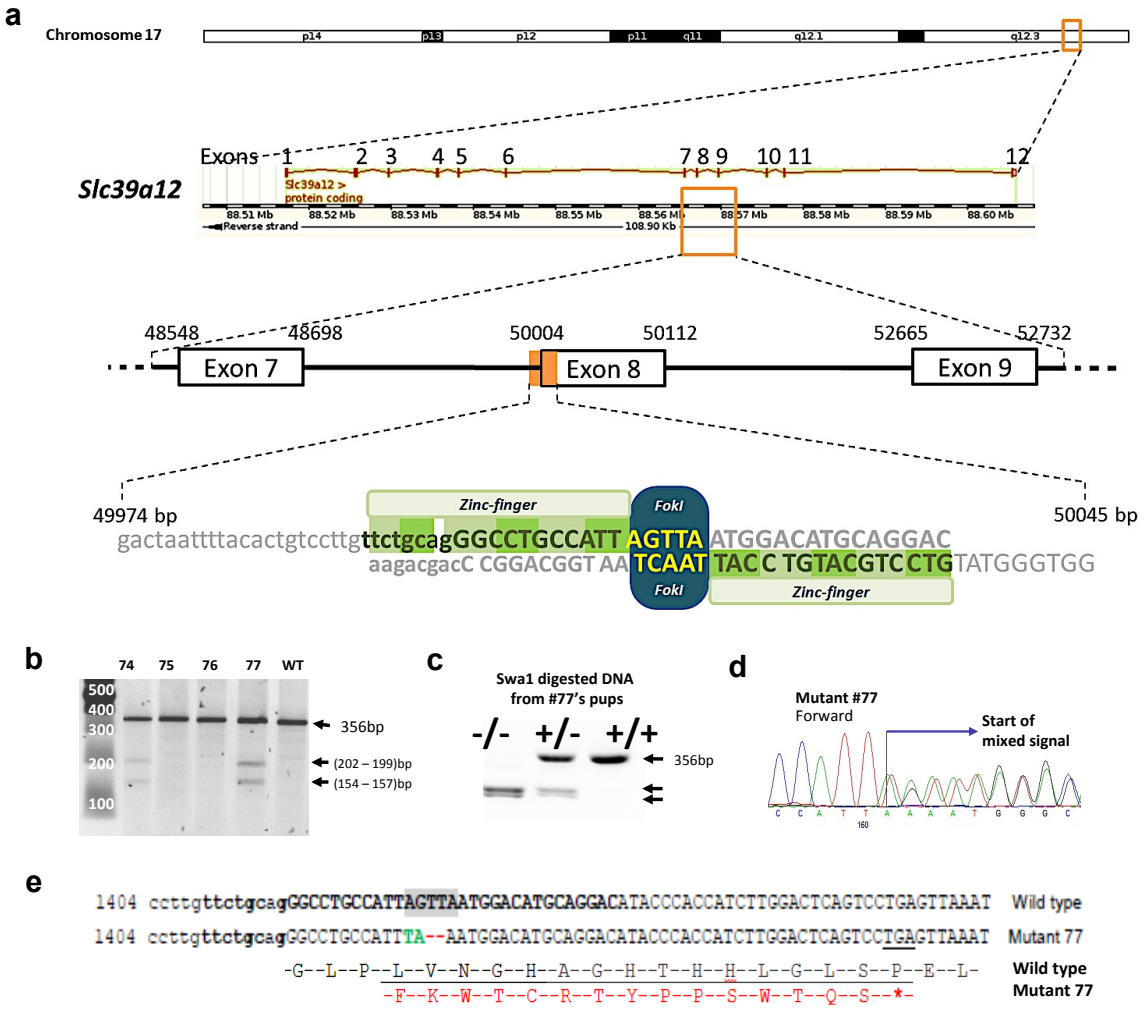

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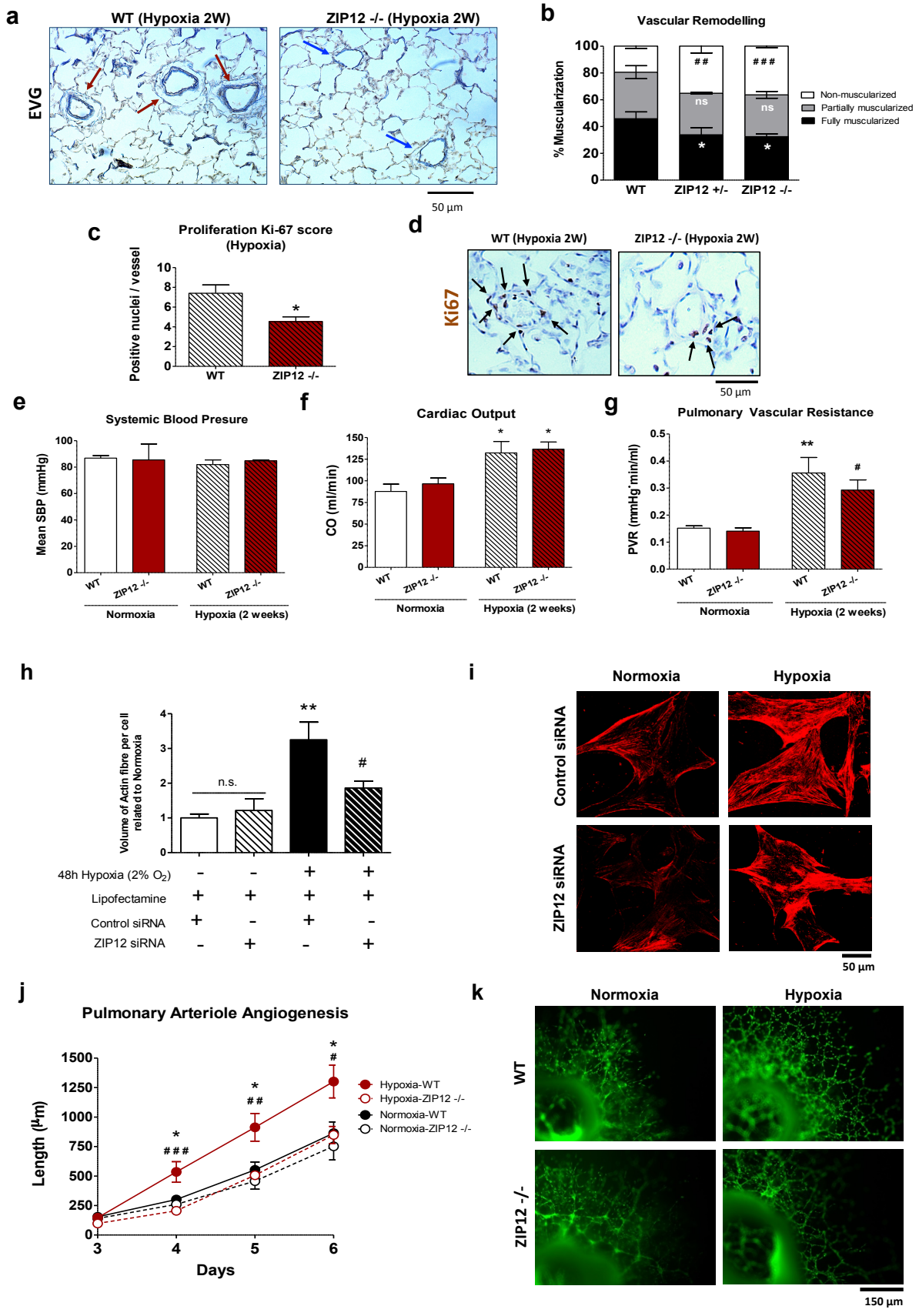

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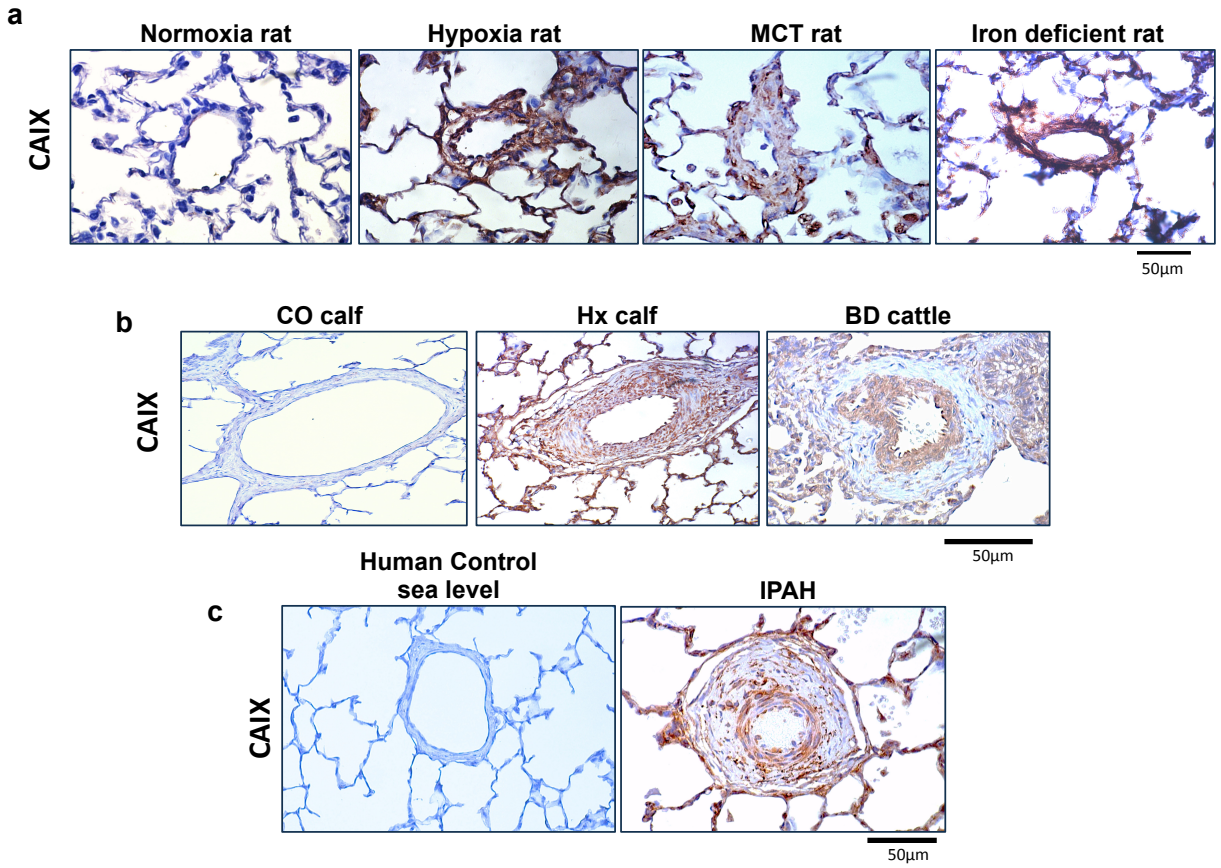

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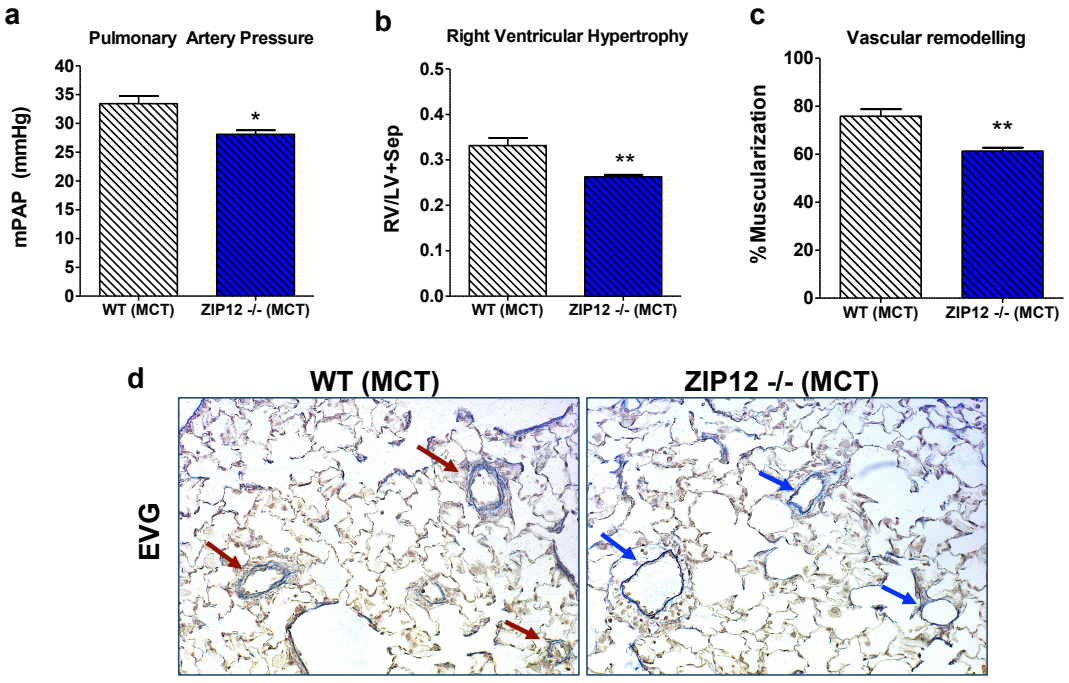

Extended Data Table 1.

					Position				Strain												
Gene Name	ENSEMBL gene ID	ENSEMBL transcript ID	SNP/Indel ID	Chromosome	Start	End	Reference Allele	Alternate Allele	F344/NCrl	FHH/EurMcwi	SHR/OlaIpcv	SHR/NHsd	SHRSP/GIa	WKY/NCrl	WKY/Sla	WKY/NHsd	Consequences	cDNA position	CDS position	Protein position	Amino acid change	Codon Change	Polyphen Prediction
Cdnf	ENSRNOG00000026493	ENSRNOT00000036299	17_85839161_G/A	17	85,839,161	-	G	A	2	0	0	0	0	6	0	0	NON_SYNONYMOUS_CODING	275	215	72	A/V	gCg/gTg	Benign
Hspa14	**ENSRNOG00000015196**	**ENSRNOT00000020384**	**17_85852829_G/C**	**17**	**85,852,829**	**-**	**G**	**C**	**2**	**0**	**0**	**0**	**0**	**0**	**0**	**0**	**NON_SYNONYMOUS_CODING**	**478**	**478**	**160**	**D/H**	**Gat/Cat**	**Probably damaging**
Hspa14	ENSRNOG00000015196	ENSRNOT00000020384	17_85853753_A/G	17	85,853,753	-	A	G	2	2	0	0	0	0	0	0	NON_SYNONYMOUS_CODING	844	844	282	T/A	Act/Gct	Benign
Hspa14	ENSRNOG00000015196	ENSRNOT00000020384	17_85853981_A/G	17	85,853,981	-	A	G	2	2	0	0	0	6	0	0	NON_SYNONYMOUS_CODING	1072	1072	358	N/D	Aat/Gat	Benign
Dclre1c	ENSRNOG00000015980	ENSRNOT00000021506	rs8173830	17	85,896,829	-	G	A	2	0	0	0	0	0	0	0	NON_SYNONYMOUS_CODING	1922	1867	623	P/S	Cct/Tct	Benign
Dclre1c	ENSRNOG00000015980	ENSRNOT00000021771	rs8173830	17	85,896,829	-	G	A	2	0	0	0	0	0	0	0	NON_SYNONYMOUS_CODING	1922	1867	623	P/S	Cct/Tct	Benign
D4A4W9_RAT	ENSRNOG00000038045	ENSRNOT00000057895	17_85993589_G/A	17	85,993,589	-	G	A	2	0	2	2	2	0	0	0	NON_SYNONYMOUS_CODING	172	170	57	R/H	cGt/cAt	Benign
F1M6A7_RAT	ENSRNOG00000038037	ENSRNOT00000057886	17_86014636_TGTA/-	17	86,014,636	86,014,639	TGTA	-	2	0	2	0	2	0	0	0	FRAMESHIFT_CODING	1063-1066	1063-1066	355-356	-	-	-
F1M6A7_RAT	ENSRNOG00000038037	ENSRNOT00000057885	17_86014636_TGTA/-	17	86,014,636	86,014,639	TGTA	-	2	0	2	0	2	0	0	0	FRAMESHIFT_CODING	901-904	901-904	301-302	-	-	-
F1M6A7_RAT	ENSRNOG00000038037	ENSRNOT00000057886	17_86014638_TA/-	17	86,014,638	86,014,639	TA	-	2	0	2	2	2	0	0	0	FRAMESHIFT_CODING	1063-1064	1063-1064	355	-	-	-
F1M6A7_RAT	ENSRNOG00000038037	ENSRNOT00000057885	17_86014638_TA/-	17	86,014,638	86,014,639	TA	-	2	0	2	2	2	0	0	0	FRAMESHIFT_CODING	901-902	901-902	301	-	-	-
F1M6A7_RAT	ENSRNOG00000038037	ENSRNOT00000057886	17_86017004_G/C	17	86,017,004	-	G	C	2	0	2	2	2	0	0	0	NON_SYNONYMOUS_CODING	1007	1007	336	P/R	cCa/cGa	Unknown
F1M6A7_RAT	ENSRNOG00000038037	ENSRNOT00000057886	17_86017414_C/A	17	86,017,414	-	C	A	2	0	2	2	2	0	0	0	NON_SYNONYMOUS_CODING	597	597	199	M/I	atG/atT	Unknown
F1M6A7_RAT	ENSRNOG00000038037	ENSRNOT00000057885	17_86017414_C/A	17	86,017,414	-	C	A	2	0	2	2	2	0	0	0	NON_SYNONYMOUS_CODING	513	513	171	M/I	atG/atT	Unknown
F1M6A7_RAT	ENSRNOG00000038037	ENSRNOT00000057886	17_86017921_-/GT	17	86,017,921	86,017,922	-	GT	0	2	0	0	0	2	2	2	FRAMESHIFT_CODING	104-105	104-105	35	-	-	-
F1M6A7_RAT	ENSRNOG00000038037	ENSRNOT00000057885	17_86017921_-/GT	17	86,017,921	86,017,922	-	GT	0	2	0	0	0	2	2	2	FRAMESHIFT_CODING	6--7	6--7	2--3	-	-	-
Nmt2	ENSRNOG00000026248	ENSRNOT00000030228	17_86060033_C/T	17	86,060,033	-	C	T	2	0	0	0	0	0	0	0	NON_SYNONYMOUS_CODING	134	134	45	S/N	aGt/aAt	Benign
Nmt2	ENSRNOG00000026248	ENSRNOT00000065609	17_86080154_-/G	17	86,080,154	86,080,155	-	G	0	5	2	2	0	2	5	2	FRAMESHIFT_CODING	86-87	86-87	29	-	-	-
Cubn	ENSRNOG00000029047	ENSRNOT00000040052	17_87738747_G/A	17	87,738,747	-	G	A	2	0	0	0	0	6	0	0	NON_SYNONYMOUS_CODING	1310	1310	437	S/L	tCg/tTg	Benign
St8sia6	ENSRNOG00000018171	ENSRNOT00000024475	17_88003467_G/A	17	88,003,467	-	G	A	2	0	0	0	0	0	0	0	NON_SYNONYMOUS_CODING	230	230	77	T/I	aCt/aTt	Benign
Slc39a12	ENSRNOG00000025639	ENSRNOT00000044313	17_88525807_C/A	17	88,525,807	-	C	A	0	2	2	2	2	2	2	2	NON_SYNONYMOUS_CODING,SPLICE_SITE	539	539	180	P/Q	cCg/cAg	Benign
Slc39a12	ENSRNOG00000025639	ENSRNOT00000066690	17_88525807_C/A	17	88,525,807	-	C	A	0	2	2	2	2	2	2	2	NON_SYNONYMOUS_CODING,SPLICE_SITE	539	539	180	P/Q	cCg/cAg	Benign
Slc39a12	ENSRNOG00000025639	ENSRNOT00000044313	17_88535671_T/G	17	88,535,671	-	T	G	0	2	2	2	2	2	2	2	NON_SYNONYMOUS_CODING	910	910	304	W/G	Tgg/Ggg	Benign
Slc39a12	**ENSRNOG00000025639**	**ENSRNOT00000066690**	**17_88535671_T/G**	**17**	**88,535,671**	**-**	**T**	**G**	**0**	**2**	**2**	**2**	**2**	**2**	**2**	**2**	**NON_SYNONYMOUS_CODING**	**910**	**910**	**304**	**W/G**	**Tgg/Ggg**	**Probably damaging**
Slc39a12	ENSRNOG00000025639	ENSRNOT00000044313	17_88575534_T/-	17	88,575,534	88,575,534	T	-	2	0	0	0	0	0	0	0	FRAMESHIFT_CODING	1628	1628	543	-	-	Truncated Protein
Slc39a12	ENSRNOG00000025639	ENSRNOT00000066690	17_88575534_T/-	17	88,575,534	88,575,534	T	-	2	0	0	0	0	0	0	0	FRAMESHIFT_CODING	1520	1520	507	-	-	Truncated Protein

Genotype code: *0 = Homozygous reference allele; 1 = Heterozygous ; 2 = Homozygous alternate allele; 6 = Ambiguous.* ***Green square*** *= shared genotype between F344 and susceptible strains;* ***Bold*** *= candidate genes;* ***Blue*** *= most important polymorphism*

Extended Data Table 2.

Rat chr 17	Genetic map		Primers		Expected size (bp)		Genotyping information			
Marker	SHRSP x BN	Physical map possition	FORWARD	REVERSE	WKY	F344	R47A	Sub A	Sub B	Sub C
D17Rat41	38.27	80234337 - 80234511	CCTTTCCCTTTCCACTCTCC	GGTAAGGGTGGTGGCAGTAG	171	157	WW	WW	WW	WW
D17Rat44	40.89	81612362 - 81612488	CAGACAAAACCCCAGCATTT	AGCAGAAAGAACCAGGCAGA	133	121	WW	WW	WW	WW
D17Got91		82277435 - 82277611	CCAGACACCAACATCACACC	CCTTCATGTGTGGAGTGTTTATG	160	176	FF	FF	WW	WW
D17Rat43	40.89	82337230 - 82337376	CACTCACTTGCTGGCTGTCT	GAGAAGAAGCTGGAGAGGCA	150	124	FF	FF	WW	WW
D17Rat42	40.33	82505422 - 82505562	TGCCGCTATTAAAAAGTAACTGC	CCAAAGGCATAAAAATCTTTCC	141	121	FF	FF	WW	WW
D17Rat62	42.35	83479938 - 83480058	GAAAGGATGGCAGGTTTTTG	TCCACAGGCTCACTGTCACT	143	121	FF	FF	WW	WW
D17Rat46	42.33	83600152 - 83600282	TGGGTTCTTTCATTCCTTGC	GCTCACCCACACACACATTC	135	125	FF	FF	WW	WW
D17Rat47	43.34	85072353 - 85072475	CCCTGCTTTCTGCTTTGAAC	TGCATATACGAATTACAGCTCAA	114	126	FF	FF	FF	WW
Del85862103		85862060 - 85862261	CACCATGAGCTCAGCAGTGT	ACACCGTCTGGCTCTCTCAG	203	193	FF	WW	FF	WW
In85923365		85923262- 85923495	ACCTTTGGCTCGGTCCTATC	AAACTTGGGTACCAGCACCA	235	243	FF	WW	FF	WW
D17Got93		86032700 - 86032904	CACTACACCTCCCAACGTCC	CTGTTGTGCCTCCTGACTAATG	227	215	FF	WW	FF	WW
D17Mit8	45.19	87465135 - 87465345	GGTCGGCATTATGGCTAAGA	CTATAGCCTCTAGGGAGGGG	193	195	FF	WW	FF	WW
D17Rat60	45.19	88268817 - 88269054	GGGGTCCAGCACTTAGCAT	GTTTTGATCATGGGGACGTT	239	241	FF	WW	FF	WW
D17Rat48	45.19	88667790 - 88667952	CACATGTCTAACTTGCCACATACA	TTTGCTGTTTCTTGTTCATGTG	166	156	FF	WW	FF	WW
Del89391756		89391713 - 89391951	TCCATGTTTTATCACCGGAAG	ATCTGATGCATGCCATAGCC	230	238	FF	WW	FF	WW
In90455808		90455697 - 90455921	AAGTTAGCCTTCCCCAAGGA	TCTGGTCTTTCCCATGTTCC	233	225	FF	WW	FF	WW
D17Rat131	47.54	93347784 - 933447990	TTAAGAAGGGCAAGCAAGGA	TCCCCATAAAAAGAAAAGGAA	203	213	FF	WW	FF	FF
D17Rat51	47.54	96587775 - 96587905	TCCCACTGGTCAATCCATTT	ACATGCAGACAGAACATTCCT	144	148	FF	WW	FF	FF